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(54) **A process for the identification of bacteria and a kit of reagents for use in this process.**

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Description

This inv ntion relates to the identification of bacteria.

Clinical bacteriology lab rat ri s are frequently called upon t test f r the presenc of pathogenic bacteria in clinical specimens and further to identify the type f bact ria concerned to help guide the clinician in the choice of treatment, e.g. antibiotic, which is to be used to combat infection.

Customary bacterial identification procedures rely upon a series of characterisation tests on the basis of which the unknown organism is assigned to a defined group of bacteria. These tests include tests in which bacteria are classified by their ability to metabolise various substrates, metabolism being determined by resultant changes in the substrate media, e.g. pH changes, which may be detected by use of coloured indicators. For such metabolic tests it is necessary, however, to grow the bacteria, usually in complete growth media, and this requires considerable expenditure of time so that it is rarely possible to identify the bacteria on the same day as the specimen arrives in the laboratory. Often, if conventional bacteria identification procedures are used, definitive identification is not possible until 48—72 hours after receipt of the specimen. In the meantime, the treatment prescribed by the clinician can at best be only a guess and may be initially incorrect, with the consequence that the infection persists and the condition of the patient deteriorates. There is a pressing need, therefore, for increase in the speed of identification of bacteria which cause infections so that the correct treatment e.g. antibiotic, may be prescribed without undesirable delay.

Very recently bacterial identification procedures have been proposed which rely only to a limited extent upon bacterial growth for identification, but depend more upon determination of enzymes which are initially present in the bacteria or are induced after a relatively short period of time, e.g. a few hours, and these tests enable bacterial identification to be made earlier than previously, sometimes on the same day as receipt of the sample. Each of these bacterial identification procedures, however, generally permits identification only of bacteria within certain limited groups and it is thus necessary to carry out a preliminary identification, usually requiring bacterial growth, to select the particular identification procedure which should be used.

A new bacterial identification procedure has now been devised which relies entirely or almost entirely upon determination of enzymes present within the organisms, and additionally advantageously provides a single identification procedure by which a very wide range of commonly encountered bacteria may be identified very rapidly.

In one aspect the present invention provides a procedure for use in the identification of bacteria, in which bacteria are subjected to a combination of tests for the determination of bacterial enzymes, characterised in that tests are carried out for the quantitative determination of each of the following enzymes (a)—(z):—

(a) lipase
(b) alpha-glucosidase
(c) beta-glucosidase
(d) beta-xylosidase
(e) beta-glucuronidase
(f) beta-galactosidase
(g) whole cell acid phosphatase
(h) acid phosphatase in the presence of agents which disrupt the bacterial cell permeability barrier
(i) DL-alanyl-beta-naphthylamine specific peptidase
(j) L-arginyl-beta-naphthylamine specific peptidase
(k) N-gamma-L-glutamyl-beta-naphthylamine specific peptidase
(l) glycyl-beta-naphthylamine specific peptidase
(m) L-4-hydroxyprolyl-beta-naphthylamine specific peptidase
(n) L-leucyl-beta-naphthylamine specific peptidase
(o) L-leucyl-4-methoxy-beta-naphthylamine specific peptidase
(p) L-lysyl-beta-naphthylamine specific peptidase
(q) L-prolyl-beta-naphthylamine specific peptidase
(r) L-pyrrolidonyl-beta-naphthylamine specific peptidase
(s) alanyl-p-nitroaniline specific peptidase
(t) glutamyl-p-nitroaniline specific peptidase
(u) diacetyl/acetoin-producing enzymes.
(v) p-nitrophenylalanine ammonia-lyase
(w) tryptophanase
(x) deoxyribonuclease
(y) glutamate decarboxylate
(z) cytochrome oxidase,

in that the tests include incubation of the bacterial sample, with substrates for the enzymes, for a period of time which, except in the case of tryptophanase and deoxyribonuclease, is suitable for the

determination of constitutive enzymes, and in that the tests, with the same exceptions, do not depend on growth of the bacteria during th test.

The procedure of the invention may be used for identificati n of a very wide range of bacteria, including most of those bacteria which are customarily encountered clinically. In particular, the procedure may be used t id ntify the commonly encountered bacterial groups: *Aeromonas, Acinetobacter, Alcaligenes, Bordatella, Citrobacter, Edwardsiella, Enterobacter, Escherichia, Flavobacterium, Hafnia, Klebsiella, Providencia, Proteus, Pseudomonas, Salmonella, Serratia Shigella, Staphylococcus* and *Streptococcus*. For example the procedure of the invention has been used to identify the following bacterial species;

Aeromonas hydrophila
Aeromonas formicans
Acinetobacter calcoaceticus var. *anitratus*
Acinetobacter calcoaceticus var. *lwoffii*
Alcaligenes faecalis
Bordatella bronchiseptica
Citrobacter freundii
Citrobacter koseri
Edwardsiella tarda
Enterobacter aerogenes
Enterobacter agglomerans
Enterobacter cloacae
Escherichia coli
Flavobacterium menigosepticum
Hafnia alvei
Klebsiella oxytoca
Klebsiella pneumoniae (sensu lato)
Klebsiella rhinoscleromatis
Providencia alcalifaciens
Providencia stuartii
Proteus mirabilis
Proteus morganii
Proteus rettgeri
Proteus vulgaris
Pseudomonas aeruginosa
Pseudomonas cepacia
Pseudomonas fluorescens
Serratia marescens
Serratia rubidaea
Serratia liquefaciens
Staphylococcus aureus
Staphylococcus epidermidis
Staphylococcus saprophyticus
Streptococcus sp

It will be appreciated, however, that the procedure of the invention may also be applied to the identification of other bacterial species besides those listed above.

The procedure of the invention permits very rapid identification of bacteria, the enzyme determination tests used generally requiring only a relatively short period of incubation, e.g. from about 10 minutes up to about 2 hours, usually from about 30 to about 90 minutes at about 40°C, to give sufficient product for detection e.g. by spectroscopic measurements. It is believed that generally the enzymes which are determined during the procedure of the invention are constitutive enzymes for the bacteria concerned, although, in two cases referred to below, the enzymes may be induced enzymes the rates of bacterial synthesis of which are very fast. Thus the tests used are generally those suitable for determination of constitutive enzymes. Without prejudice to the foregoing, however, determination of tryptophanase and deoxyribonuclease appears to require bacterial growth, usually requiring incubation for a period of 2—2½ hours.

Generally the tests which are used in the procedure of the invention for determination of bacterial enzymes may comprise any suitable tests for the determination of the enzymes in question which are characteristically not dependent on growing the organisms during the test, and which may include those non-growth enzyme determination tests for the enzymes in question which are well known in the art.

These tests are usually of the kind in which the enzyme is determined by its ability to interact with a specific substrate. Interaction of the enzyme with the substrate, on incubation, usually gives ris to a

product which may be quantitatively determined either directly or after further treatment which may include chemical synthesis from th initial nzym product.

The product of the enzyme interaction may be detected by spectrometric measurements including fluorimetry or c lorimetry. F r example, the specific enzyme substrate may comprise an umbelliferyl derivative which on interaction with the enzyme gives rise to umbelliferone which is monitored fluorimetrically, or the substrate may comprise a nitrophenyl, nitroaniline or similar type of derivative which on interaction with the enzyme gives rise to a coloured product which is monitored colorimetrically.

An example of an enzyme which may be determined by spectrometric measurement of the direct product of enzyme interaction with a substrate is cytochrome oxidase; for instance, by interaction of sample with tetramethyl-p-phenylene-diamine (TMPD), an indicator which is oxidised by cytochrome oxidase, a purple coloration is given when cytochrome oxidase is present in the sample. Also, acid phosphatases, beta-galactosidases and phenylalanine deaminases may be determined spectrometrically, using for instance, nitrophenyl derivatives, although it is normally necessary to treat the enzyme-substrate mixture with alkali after incubation so as to develop the nitrophenol coloration by raising the pH above the pH optimum of the enzyme reaction.

Products which require further treatment after enzyme reaction with the substrate before monitoring, may be detected spectrometrically. For example, ammonia-releasing enzymes, such as leucine deaminase may be determined by reacting the ammonia produced by the enzyme interaction with the colour-producing reagent such as the Nessler reagent, and monitoring the resultant colour by colorimetric measurement. The ammonia released may be measured directly in the enzyme-substrate reaction mixture or may be removed, e.g. by dialysis, from the reaction mixture before assay.

Also, for example, diacetyl-producing or acetoin-producing enzymes may be determined by spectrometric monitoring of enzyme products after further treatment. Such enzymes can be determined by the Voges-Proskauer technique for testing for the presence of diacetyl/acetoin. The technique does not differentiate between the production of acetoin ($CH_3COCHOHCH_3$) and its oxidation product diacetyl ($CH_3COCOCH_3$).

Furthermore, glutamate decarboxylase activity may be determined by colorimetrically determining the carbon dioxide released as a result of interaction of the enzyme with glutamic acid. For instance, carbon dioxide is determined colorimetrically by dialysis out of the reaction mixture after addition of acid, e.g. H_2SO_4, through a hydrophobic membrane into a buffered indicator which changes colour as a result of the change in acidity due to the CO_2.

Other enzymes may be determined by suitable techniques; for instance, substrate specific peptidases are determined by use of naphthylamine or nitroaniline derivatives, naphthylamine produced being determined either directly by fluorimetry or by colorimetry after diazonium coupling.

The tests used for determination of the enzymes may be varied as desired, for instance to increase the organism specific selectivity of the tests. Thus, two tests for determination of phosphatase activity are included within the procedure of the invention, one for whole cell acid phosphatase activity and one for acid phosphatase activity in the presence of agents which disrupt the bacterial cell permeability barrier. Any suitable agent may be used, though Cetrimide (cetyl trimethylammonium bromide) and lysozyme, especially in combination, are particularly preferred. The use of such an agent, for instance, has the effect of selectively decreasing the acid phosphatase activity of cells of *Proteus* bacteria and increasing that of *Klebsiella* cells.

The invention includes kits of reagents for use in the procedure of the invention. Such kits typically comprise separate specific substrates for each of the enzymes which it is desired to determine in the procedure of the invention. Thus, for example, a basic kit for use in the procedure of the present invention comprises separate specific substrates, for determination of (a)—(z) listed previously. Preferably these substrates are such as to give chromogenic or fluorogenic products on interaction with corresponding enzymes to advantageously permit colorimetric or fluorimetric monitoring. Additionally the kits may also comprise suitable buffer solutions and other reagents, e.g. colour-developing or fluorescence-developing reagents.

The procedure of the present invention is generally applicable to the identification of bacteria in clinical specimens including urine samples, throat swabs and sputum, wound swabs, stools, and blood samples. Bacteria may be isolated from the specimens prior to identification. For example, bacterial cultures are prepared from the specimens and colonies of the organisms to be identified are harvested from the cultures after a sufficient period of growth, e.g., normally 18 hours, and made up into a suitable form, e.g. suspension form, for determination by the procedure of the invention. In particularly preferred embodiment, however, it is envisaged that the procedure of the invention will be carried out on samples derived directly from clinical specimens, for instance, on samples derived directly from urine samples, without need for growth of bacteria and isolation of single colonies.

Prior to assay of bacterial enzymes, however, the samples containing bacteria, whether derived directly from clinical specimens or derived as single colonies after bacterial growth, may in some cases be subjected to treatment to disrupt the permeability barrier of the bacteria and release the enzymes for assay. Any suitable treatment may be used to disrupt the bacterial permeability barrier. Generally during determination of cytoplasmic and periplasmic enzymes, such as beta-galactosidases and acid

phosphatases, such prior disruption of the bacterial permeability barri r may be desirable, though the other enzymes, such as deaminases, which appear to be membran -associated may require the bacterial cells to be kept intact for nzyme activity to be maintained.

The enzyme assay tests of the procedure f the invention may be carried out by any suitable meth d or means, including continuous flow and discrete sampl analysis t chniques, such as those which are well known in the art. In one mbodim nt a discrete analyser, such as the Kem-O-Mat system, is used. In another embodiment the enzymes may be assayed by automated continuous flow analysis techniques. In such continuous flow analysis methods it may be desirable to include a protein determination in view of the differing protein concentrations of various bacteria, so that the specific enzyme activities of the bacteria may be determined. Also a protein assay may provide a measure of the blank in spectrometric assays for the absorbance due to the concentration of the organisms.

The conditions used during enzyme determinations may be varied as desired. For example, in continuous flow analysis techniques the relative organism to reagent concentration may be raised, e.g. a sample to reagent ratio in the range from 1:3 up to 3:1, to increase the levels and thus permit detection of enzyme at lower bacterial suspension concentrations. Also, preferably, relatively elevated temperatures, e.g. temperatures of 40°C, may be used during incubation of sample and substrate to increase the rate of interaction. Preferably, using continuous flow techniques, it is possible to achieve bacterial identification at a very early stage, especially within 1 hour of the sample reaching the laboratory, e.g. if apparatus comprising a single channel for each enzyme test is employed.

In further preferred embodiments, however, the procedure of the invention may be carried out using a test card or other suitable apparatus comprising a plurality of wells or compartments which separately contain specific enzyme substrates for each of the enzyme tests of the procedure and comprising other reagents, as required, e.g. colour-developing reagents. In use, the sample, usually bacterial suspension, is added to each component and a detectable product is developed after a relatively short incubation period e.g. from 20 minutes up to 2 hours in preferred embodiments, and if necessary after addition of colour-developing reagents. The amount of the corresponding enzymes present in the bacterial sample is then determined. Such apparatus is included within the scope of the invention, and in particularly preferred embodiments may be adapted to handling by automated techniques including automated, preferably computerised, spectrometric scanning techniques which identify the bacterial species directly from the responses of the enzyme tests.

The procedure of the invention may incorporate additional tests for determination of bacterial enzymes besides those mentioned previously as tests (a)—(z); for instance to strengthen the identification of certain groups of bacteria. Thus, the procedure may include a test for determination of bacterial urease activity.

In a second aspect the invention also includes a bacterial identification procedure for use in the rapid differentiation of the commonly encountered bacterial groups *Escherichia, Klebsiella spp, Proteus spp* and *Pseudomonas spp*, in which a sample comprising bacteria of one of these groups is subjected to a combination of tests for determination of enzymes, characterised in that tests are carried out for the determination of each of the following enzymes: acid phosphatase in the presence of an agent which disrupts the bacterial cell permability barrier, diacetyl/acetoin-producing enzymes, beta-galactosidase, glutamate decarboxylase, phenylalanine deaminase, cytochrome oxidase and urease. Again, the bacterial sample is incubated, with substrates for the enzymes for a period of time suitable for the determination of constitutive enzymes, examples of which are given above, and the tests do not depend on growth of the bacteria during the test. This more limited combination of seven tests may be used to rapidly differentiate the bacterial groups *Escherichia, Klebsiella spp, Proteus spp* and *Pseudomonas spp*, substantially as hereinbefore described with reference to the full identification procedure incorporating the 26 tests (a)—(z).

The invention also includes kits for use in this 7 test procedure, the basic kit typically comprising separately specific substrates for acid phosphatase, diacetyl/acetoin-producing enzymes, beta-galactosidase, glutamate decarboxylase, phenylalanine deaminase, cytochrome oxidase and urease activity. The kit may also comprise colour-developing or fluorescence-developing reagents and other optional components as for the 26-enzyme test kits.

In general the procedures of the invention rely upon determination of the enzyme activity profiles of the bacteria undergoing identification, and in accordance with the invention the combination of enzyme tests chosen, i.e. tests (a)—(z) or the limited combination of 7 tests, typically gives a unique "fingerprint" for the bacteria. The unique "fingerprint" for each species or group of bacteria may be determined with reference to the enzyme activity profiles of previously identified bacteria; for instance, from bacteria obtained from culture collections. Enzyme activity profiles are determined quantitatively. The use of data-processing techniques are desirable to facilitate the identification by comparison of enzyme profiles of unknown bacteria with those of previously identified bacteria. For example, processing of results obtained by discriminant function analysis, e.g. using the SPSS package (Statistical Package for Social Sciences) has been found to be particularly useful.

The procedures of the invention typically permit very rapid identification of bacteria, a single procedur only being required for identification of a very wide range of bacteria, including most of those

bacteria which are commonly encountered clinically. This provision of a single procedure advantageously obviates the need f r a preliminary test and th undesirable delay that this causes.

The invention is further described by way of illustration only in the following description and examples which refer to the acc mpanying drawings:

Figure 1 which is a diagrammatic repres ntation of a manifold used for continuous flow analysis of nitrophenol-releasing enzymes in the procedure of the invention;

Figure 2 which is a diagrammatic representation of a similar manifold for analysis of ammonia-releasing enzymes;

Figure 3 which is a diagrammatic representation of a similar manifold for analysis of diacetyl/acetoin-producing enzymes;

Figure 4 which is a diagrammatic representation of a similar manifold for assay of cytochrome oxidase and protein;

Figure 6 which is a diagrammatic representation of a flow chart for a three-channel combination continuous flow analysis system for carrying out the procedure of the invention and comprising the manifolds of Figures 1 to 5, and

Figure 7 which is a diagrammatic representation of a manifold used for continuous flow analysis of enzymes using substrates which release fluorescently active products.

Tests for determination of bacterial acid phosphatase (in the presence of an agent which disrupts the bacterial cell permeability barrier), beta-galactosidase, glutamate decarboxylase, leucine deaminase, phenylalanine deaminase, cytochrome oxidase, urease and diacetyl/acetoin-producing enzymes are carried out by continuous flow analysis techniques using a three-channel combined system, a flow chart for which is given a Figure 6, comprising enzyme determination manifolds as shown diagrammatically in Figures 1 to 5.

The various enzyme determinations are carried out as follows:—

Enzymes utilising nitrophenyl substrates (beta-galactosidase, phenylalanine deaminase and acid phosphatase).

The manifold shown in Figure 1 is used for determination of enzymes utilising nitrophenyl substrates, i.e. beta-galactosidase, phenylalanine deaminase and acid phosphatase.

A stream of organism suspension 1 is mixed with an air-segmented buffer stream 2 in the first single mixing coil (SMC) 3, and is then mixed with the substrate 4 in the second SMC 5 and incubated for 18 minutes in a glass coil maintained in an oil bath 6 at 40°C. The reaction is stopped by addition of a strong alkaline solution 7 (1.9M NH_4OH, 0.68M NaOH, Triton-X-100 0.3 g/l) which also acts as a colour developer for the released p-nitrophenyl molecule. The stream is then de-bubbled and passed through a 20 mm flow cell in a photometer 8 and the absorbance measured at 405 nm for beta-galactosidase and acid phosphatase and at 480 nm for phenylalanine deaminase. The absorbance readings obtained are registered on a recorder 10. The figures given in the enclosed area 11 in Figure 1 are the flow rates used for the various reagent and reactant streams.

The various buffer solutions and substrates used are given below in Table 1.

TABLE 1
Enzyme systems tested with nitrophenyl substrates

Enzyme	Substrate	Buffer		
		Iron	pH	Additions
Acid phosphatase	p-nitrophenyl-phosphate 1.5 mM	0.1M Na acetate	5.6	200 micrograms/ml of cetrimide and lysozyme
Beta-galactosidase	p-nitrophenyl-beta-D-galacto-pyranoside 0.5 mM	0.1M K phosphate	7.4	200 micrograms/ml of cetrimide and lysozyme
Phenylalanine deaminase	DL-beta-(p-nitro-phenylalanine 0.5 mM	0.1M K phosphate	8.0	None

It is believed that the interaction of phenylalanine deaminase with the DL-beta-(p-nitrophenyl)-analine substrate gives rise to production of p-nitrophenylpyruvic acid which is the product which is measured by absorbance at 480 nm.

Ammonia-releasing enzymes (leucine deaminase and urease)

With reference to Figure 2 the method used for assay of ammonia-releasing enzymes, i.e. leucine

deaminase and urease, is based on that of Bascomb and Grantham (1975 "Some Methods for Microbiological Assay" ed. Board & Lovelock pp 20—54, Academic Press, N.Y.) but using different sized tubes. Ammonia released from the substrates is assayed by adding the Nessler reagent to ammonia which has been collected by dialysis into a 0.005 M HCl recipient stream. Attempts to measure the ammonia directly in the organism/substrate stream are not satisfactory due to troublesome base-line drift and non-reproducibility f the standards. Urease activity is determined in the absence of Tris, maleate or phosphate buffer as their presence caused a noisy base-line the enzyme being measured in the presence of distilled water only (pH approximately 4.7—5.0). HC_4Cl solutions are used as standards in both assays. The substrates used are 5 mM L-leucine in 0.5M phosphate borate, pH 8.0 and 100 mM urea in fresh distilled water. The Nessler reagent is prepared as described by Bascomb and Grantham in the above-mentioned publication and diluted 1:10 in fresh glass distilled water daily. A 20 mm flow cell is used in the photometer and absorbance is measured at 420 nm.

In Figure 2 the functions of the various components and the relevance of the information given is similar to Figure 1. The broken line arrow 12 indicates the direction of flow by-passing across the dialyzer 13 without dialysis.

Diacetyl/acetoin-producing enzymes

The Voges-Proskauer reaction for the presence of acetoin/diacetyl is used, in the manifold illustrated in Figure 3, for determination of diacetyl/acetoin-producing enzymes. The method of measurement used is developed from that described by Kamoun *et al.* (Clin. Chem. 1972, *18*, 355—357). The substrate solution contains 0.3M sodium pyruvate, 0.1M acetate buffer (pH 4.5), 0.1 mM thiamine pyrophosphate and creatine 2 g/l) in 2M sodium hydroxide. Diacetyl solutions are used as standards. Absorbance is measured in the red region of the spectrum 525 nm using a 20 mm flow cell.

Glutamate decarboxylase

Glutamate decarboxylase is determined, with reference to Figure 4, by interaction with substrate comprising glutamate to produce CO_2 which is dialysed from the reaction mixture into a buffered cresol red solution where its presence causes a colour change in the indicator. The method used is based on those described by Leclerc (Annls. Inst. Pasteur, Paris (1967) *112*, 713—731), Moran and Witter (1976 J. Food Sci. *41*, 165 167) and Technicon Methodology AA 11—08. Technicon sodium carbonate standards are used. The substrate used comprises 0.1M acetate buffer (pH 3.8), 0.05M sodium glutamate and pyridoxal phosphate 20 mg/l. After incubation of the sample and substrate 0.5M sulphuric acid diluent, containing Brij-35 (30% Technicon) 1 ml/l, is added to lower the pH and assist removal of CO_2 produced by dialysis through a hydrophobic dialysis membrane. The colour reagent used contains 0.4 mM Tris, ammonia solution 28 microlitres/litre, Brij-35 20 microlitres/litre and cresol red 20 micrograms/litre. Absorbance is measured at 420 nm in a 10 mm flow cell.

Cytochrome oxidase and protein assays

Cytochrome oxidase and protein are assayed using the manifold apparatus illustrated in Figure 5—which contains similar information and components having similar functions as in Figures 1 to 4.

The reagents used for assay of cytochrome oxidase activity are 0.05M Tris-maleate buffer, pH 6.0, introduced prior to the first single mixing coil 0.5 mM, N,N,N',N'-tetramethyl-p-phenylene diamine dihydrochloride in 0.001% (w/v) ascorbic acid introduced prior to the second single mixing coil. The incubation period used is 17 minutes at room temperature and absorption is measured at 550 nm in a 10 mm flow cell.

Protein assay

The protein assay used is based on that of Lowry *et al.* (1951, J. Biol. Chem. *193*, 265—275) and as described by Bascomb and Grantham in the above-mentioned publication, though using smaller tubes. The reagents used are alkaline copper solution prepared daily by mixing 2 ml sodium potassium tartrate (10 g/l) with 2 ml $CuSO_4 \cdot 5H_2O$ (5 g/l) and 46 ml 0.2M NaOH containing 0.37M Na_2CO_3 (anhydrous), in the order described. The alkaline copper reagent is introduced prior to the first single mixing coil. Folin and Ciocalteu's reagent (BDH) is diluted 1:8 in distilled water daily and introduced prior to the second single mixing coil. The incubation period used is 17 minutes at room temperature and absorption is measured at 660 nm in a 10 mm flow cell. Bovine serum albumin solutions are used as standards. A few drops of chloroform are added to the sodium tartrate, carbonate and protein standard solutions to prevent microbial contamination.

The enzyme assays described above are carried out in a combined continuous flow analysis system, the flow chart of which is illustrated in Figure 6, comprising three channels which are run simultaneously: one channel (A) for assaying enzymes utilising nitrophenyl derivatives, the second channel (B) for assaying protein and cytochrome oxidase, and the third channel (C) for assaying diacetyl/acetoin-producing, ammonia-producing and CO_2-producing (glutamate decarboxylase) enzymes. The NH_3 and CO_2 products are dialysed from the reaction mixtures and the Nessler and 1-naphthol colour-developing reagents added, where appropriate, between the incubation coil and the third SMC. The stream from the single sample probe is divided into three, providing bacterial

suspensions to each of the channels A, B and C. Th bacterial suspensions are maintained in cups on a revolving sample plate D, while substrat s and buffer solutions are provided on continuous streams via the tubes.

All bacterial suspensi ns are first tested for activity of three enzymes, one in each of the channels A, B and C. When th cycle is complete, the substrate, buffer and sample lines are transferred manually to reagents for the next batch of three tests and the sampling of bacteria is restarted. This proc ss is repeated four times, including control runs with distilled water in the substrate lines to obtain the absorbance values of bacterial suspensions in the acid phosphatase, cytochrome oxidase, leucine deaminase and urease assays.

Example 1

A total of 199 suspensions of different bacteria are tested by the procedure outlined above using the continuous flow apparatus illustrated in the accompanying drawings. The bacteria are isolated from routine urine specimens cultured overnight at 37°C on MacConkey agar plates (Difco Laboratories or Tissue Culture Services). Only plates that show a homogeneous colony appearance are used.

Bacterial suspensions each comprising ten colonies in 5 ml saline are prepared in a separate location and are brought to the automated system to ensure that automated identification is carried out without any knowledge of the colonial appearance of the samples. A 0.1 ml aliquot is removed from each suspension and added to 5 ml nutrient broth which is then incubated at 37°C for 2 hours on a Matburn rotary mixer. These suspensions are used for inoculation of a chosen set of conventional test media and a nutrient agar slope to be kept for further testing. The remaining saline suspension is tested directly by the automated procedure. Cetrimide and lysozyme are included in the buffer solutions for acid phosphatase and beta-galactosidase assays and mixed with the saline organism suspensions for $2\frac{1}{2}$ minutes at room temperature prior to addition of the substrate to effect disruption of the bacterial permeability barrier. This treatment halved the absorbance at 340 nm of all gram-negative bacteria tested.

The results obtained using the automated procedure are given below in Table 2 and are compared in Table 3 with results obtained by conventional techniques from which the bacteria are identified following the method of Cowan and Steel (1974 "Manual for the identification of Medical Bacteria" 2nd Edition, University Press, Cambridge).

TABLE 2
Distribution of positive results (%) in automated tests according to the invention

	E. coli	*Klebsiella spp*	*Proteus spp*	*Pseudomonas spp*
Number strains	125	39	16	19
Automated tests				
Acid phosphatase	1	95	100	0
Diacetyl/acetoin-producing (Voges-Proskauer)	0	51	0	0
Beta-galactosidase	94	82	0	0
Glutamate decarboxylase	99	0	62	0
Leucine deaminase	0	8	94	0
Cytochrome oxidase	0	0	0	95
Phenylalanine deaminase	2	0	100	0
Urease	0	62	75	10

It is evident from Table 2 that leucine deaminase can be omitted, since it performs substantially the same function as phenylalanine deaminase.

Example 2

A total of 96 suspensions of different organisms is tested by the automated method described above in Example 1 and the preceding description, except in this case each suspension comprises a single colony of organisms in 1 ml of saline. The automated test procedure adopted is the same as

Example 1, though with fewer control runs, and similarly conventional tests are carri d out on aliquots of suspension for the sake of comparison. The results obtained, in terms of the success rate, are given b low in Table 3 which also includes results for the success rate of the automated tests of Example 1.

TABLE 3

Agreement between automated and conventional identification

	E. coli	*Klebsiella spp*	*Proteus spp*	*Pseudomonas spp*	Total
Number of strains tested					
Example 1	125	39	16	19	199
Example 2	61	22	3	5	96
Correct identification by automated assay (%)					
Example 1	99	97	100	95	98
Example 2	100	100	100	100	100

As can be seen, the agreement rate achieved with the automated system in Example 1 is 98%. The three strains that are not identified include a strain of *Klebsiella spp* showing only acid phosphatase activity, a strain of *E. coli* showing only beta-galactosidase activity, and a strain of *Pseudomonas spp* for which the cytochrome oxidase activity results are not available. All three organisms are originally classified as unidentified and on repeat testing are identified correctly. Thus Table 3 indicates that it is possible to correctly identify all four bacterial groups, namely *Escherichia, Klebsiella spp, Proteus spp* and *Pseudomonas spp*, by the automated testing procedure.

The success rate achieved in Example 2 was as good as that in Example 1. Thus it is possible to identify some bacteria from a single colony by the above automated tests.

Example 3

A total of 304 suspensions of culture collection and freshly isolated strains were tested by the full 26 test procedure of the invention as described below.

Culture collection strains were from the National Collection of Type Cultures, the Computer Trial Laboratory at Colindale and the Bacteriology Department, St. Mary's Hospital Medical School; all had been characterised and identified by conventional methods. Fresh strains were isolated from routine urine specimens and identified using API strip 20E. All strains were cultured overnight at 37°C on MacConkey agar plates, without added sodium chloride (Difco Laboratory and Tissue Culture Services). Bacterial suspensions of 8 colonies in 4 ml sterile saline were used for all enzyme assays.

Enzyme activities were measured using 3 different analytical systems. The continuous flow system, as described in Example 1, was used to determine activity of cytochrome oxidase, glutamate decarboxylase and protein content. A semi-automated continuous flow method was used for detection of enzymes listed in Table 4, by fluorimetry using the continuous flow manifold shown in Figure 7. A discrete analyser (Kem-O-Mat) was used to perform the tests listed in Table 5.

In the semi-automated method buffered substrate solution (200 microlitres) and bacterial suspension (200 microlitres) were added manually to Auto Analyser cups. The bacteria and substrate mixture was incubated for 90 minutes at 40°C and the fluorescence measured in a Lochart fluorimeter attached to a Newton sampler, using a continuous flow manifold as detailed in Figure 7. This system allowed measurement of 100 samples per hour. The enzymes tested and the composition of the reagents are given in Tables 4(a) and 4(b).

TABLE 4(a)—Hydrolyases

Enzyme	Buffer	Buffered substrate Substrate	C ncn. mM	Manifold reagent
Lipase	Tris-HCl pH 9.0, 0.05M	4-methylumbelliferyl-nonanoate	0.05	HCl, 20 mM
Alpha-glucosidase	Tris-phosphate pH 8.0, 0.1M	4-methylumbelliferyl-alpha-D-glucopyranoside	0.2	HCl, 20 mM
Beta-glucosidase	Tris-phosphate pH 8.0, 0.1M	4-methylumbelliferyl-beta-D-glucopyranoside	0.5	HCl, 20 mM
Beta-xylosidase	Tris-phosphate pH 8.0, 0.1M	4-methylumbelliferyl-beta-D-xylopyranoside	0.2	HCl, 20 mM
Beta-glucuronidase	sodium acetate pH 5.6, 0.1M	4-methylumbelliferyl-beta-D-glucuronide	0.5	glycine buffer pH 8.8, 1 mM

All substrates were supplied by Koch Light. They were dissolved in methoxyethanol and diluted with appropriate buffer to the final concentrations listed.

TABLE 4(b)—Substrates used for peptidase assays

Substrate	Supplier
DL-Alanine-beta-naphthylamide hydrochloride	Sigma
L-Arginine-beta-naphthylamide hydrochloride (arg-NAP)	Sigma
N-Gamma-L-glutamyl-beta-naphthylamide	Sigma
Glycyl-beta-naphthylamide hydrochloride	Koch Light
L-4-Hydroxyprolyl-beta-naphthylamide (OH-pro-NAP)	Sigma
L-Leucyl-beta-naphthylamide hydrochloride (leu-NAP)	Sigma
L-Leucyl-4-methoxy-beta-naphthylamide hydrochloride (leu-4-m-NAP)	Sigma
L-Lysyl-beta-naphthylamide carbonate	Koch Light
L-Prolyl-beta-naphthylamide hydrochloride (pro-NAP)	Sigma
L-Pyrrolidonyl-beta-naphthylamide (pyr-NAP)	Sigma

All substrates were dissolved in dimethyl sulphoxide and diluted to a final substrate concentration of 0.1 mM with 0.1M Tris-phosphate buffer, pH 8.0, containing cobaltous nitrate (2 micromoles). The reagent used in the continuous flow manifold was 0.1M Tris-phosphate buffer, pH 8.0.

The discrete analyser, Kem-O-Mat, (Coulter Electronics) was used to perform the tests listed in Table 5. The bacterial suspensions were placed in the sample cups, buffers were dispensed by the diluent syringe, substrates and reagents (for developing the colour of the reaction end-products) were dispensed by the reagent syringe. To increase sample throughput and lengthen incubation periods, cuvettes containing bacterial suspensions, buffer and substrate were incubated outside the Kem-O-Mat. The cuvette changer was used for simultaneous insertion of all 32 cuvettes and for their removal. Two program cartridges were used to perform each test. Program cartridge 1 was used for distribution of bacterial suspensions, buffer, cofactors and substrate to each cuvette and for reading the initial absorbance of each cuvette. The cuvettes were then removed and placed in a 40°C incubator. The diluent and reagent syringes and probes were rinsed and charged with the reagents for the next enzyme test. After incubation the cuvettes of each enzyme test were returned to the analyser. Program cartridge 2 was used for adding the second reagent and for reading the final absorbance. The details of reagent composition are given in Table 5. For all tests 305 microlitres f dilu nt and 70 microlitres of

reagent 1 were dispensed into each cuvette. Bacterial suspensions were dispensed into each cuvette. Bacterial suspensions were dispensed in 50 microlitres aliquots for the DNAase test and in 25 microlitres aliquots for all other tests. Minimum incubation periods for tryptophanase and DNAase were 2 hours, for VP and PNPA $1\frac{1}{2}$ h urs, and for the remaining tests 1 hour. At the end of the incubation period reagent 2 was added to the cuvettes c ntaining substrat and bact rial suspension in 50 microlitres aliquots for the VP, PNPA, PNPP, PNPP+C+L and PNPG+C+L tests and in 350 microlitres aliquots for the INDOLE test. Final absorbance was read 2 minutes after addition of the second reagent, except for VP and INDOLE tests which were read after 20 minutes. Absorbance measurements from the 3 different analytical systems were fed into a computer for calculation of enzyme activity and specific enzyme activity of each organism suspension in each enzyme test.

TABLE 5 (1)

Test	Diluent*	Reagent 1	Reagent 2
Diacetyl/acetoin producing enzymes (V-P reaction) (VP)	0.05M acetate buffer, pH 4.5, containing 0.15M sodium pyruvate, creatine, 1 g/l and NaCl, 4.25 g/l	0.4 mM cocarboxyl-ase	2M NaOH containing alpha-naphthol, 50 g/l
p-Nitrophenylalanine ammonia-lyase (PNPA)	0.1M potassium phosphate buffer, pH 8.0	10 mM DL-beta-(p-nitrophenyl)alanine	1.9M ammonium hydroxide containing 0.68M NaOH and Triton-X-100, 0.3 g/l
Alanyl-p-nitroaniline specific peptidase (PNAA)	0.1M potassium phosphate buffer, pH 8.0	3.5 mM L-alanine-4-nitroanilide hydro-chloride (BDH)	—
Glutamyl-p-nitro-aniline specific peptidase (PNAG)	0.07M Tris-phosphate buffer, pH 8.0, contain-ing cobaltous nitrate, 2 micromoles	3.5 mM gamma-L-glutamyl 4-nitro-anilide	—
Acid phosphatase (PNPP)	0.03M sodium acetate buffer, pH 5.6, contain-ing NaCl, 2.8 g/l	5 mM p-nitrophenyl-disodium phosphate	1.9M ammonium hydroxide containing 0.68M NaOH and Triton-X-100, 0.3 g/l
Acid phosphatase (PNPP+C+L)	0.03M sodium acetate buffer, pH 5.6, contain-ing cetrimide, 0.007 g/l; lysozyme, 0.007 g/l; and NaCl, 2.8 g/l	5 mM p-nitrophenyl-disodium phosphate	1.9M ammonium hydroxide containing 0.68M NaOH and Triton-X-100, 0.3 g/l
Beta-galactosidase (PNPG+C+L)	0.03M potassium phosphate buffer, pH 7.5 contain-ing cetrimide, 0.007 g/l; lysozyme, 0.007 g/l; and NaCl, 2.8 g/l	8 mM p-nitrophenyl-beta-D-galacto-pyranoside	1.9M ammonium hydroxide containing 0.68M NaOH and Triton-X-100, 0.03 g/l
Tryptophanase (INDOLE)	Peptone (Oxoid L37), 5 g/l and NaCl, 6.75 g/l in distilled water	2mM L-tryptophan	78% (v/v) ethanol con-taining 1.16M HCl and p-dimethylaminocinnam-aldehyde, 2.37 g/l
Deoxyribonuclease	0.07M Tris-HCl buffer, pH 9.0, containing $MgCl_2$ 20 mM; ethidium bromide, 23 mg/l; and deoxyribo-nucleic acid (Sigma calf thymus), 227 mg/l	distilled water	—

*All diluent solutions contain Triton-X-100, 0.1 ml/l, to decrease air bubbles on cuvette walls.

Analysis of results

Three hundred and four cultures falling into 35 species w r tested by both Kem-O-Mat and continuous flow systems on a total of 26 t sts and a protein assay. Thirty-one strains were excluded from the calculations because of missing values, mixed cultures and doubtful identity by conventional testing. The remaining 273 cultures w r divided into a kn wn s t (Training Set) comprising of between 210 and 221 cultures of mainly culture collection strains; and an unknown set (Test Set) of 52—73 cultures, mainly of strains freshly isolated from cultured urine specimens and tested the day after the urine specimen was received.

The discriminant function analysis (DFA) using SPSS software was used to identify both training and test sets.

The 35 species were divided either into 17 genera or sub-divided within each genus to species or groups of species as shown in Table 6.

TABLE 6
Division and coding of bacterial taxa in discriminant function analysis (DFA)

	Coding used in DFA					
	Number of groups					
Bacterial taxon	17	22A	22B	25A	25B	35
Escherichia	1	1	1,2	1,2	1,2	1
Klebsiella pneumoniae sensu lato	2	2	3	3	3	2
Klebsiella rhinoschleromatis	2	2	3	4	4	3
Klebsiella oxytocum	2	2	3	3	3	4
Proteus mirabilis	3	3	4	5	5	5
Proteus morganii	3	3	4	6	6	6
Proteus rettgeri	3	0	0	0	0	7
Proteus vulgaris	3	3	4	7	7	8
Pseudomonas aeruginosa	4	4	5	8	8	9
Pseudomonas cepacia	4	5	6	9	9	10
Pseudomonas fluorescens	4	6	7	8	8	11
Staphylococcus epidermedis	5	7	8	11	11	12
Staphylococcus aureus	5	7	8	10	10	13
Staphylococcus saprophyticus	5	7	8	11	11	14
Streptococcus	6	8	9	12	12	15
Citrobacter freundii	7	9	10	13	13	16
Citrobacter koseri	7	10	11	14	14	17
Providencia alkalifaciens	8	11	12	15	15	18
Providencia stuartii	8	11	12	15	15	19
Serratia marcescens	9	12	13	16	16	20
Serratia rubidaea	9	12	13	16	16	21
Serratia liquefaciens	9	12	0	16	0	22
Acinetobacter calcoaceticus va. anitratus	10	13	14	17	17	23

TABLE 6 (contd.)

Division and coding of bacterial taxa in discriminant function analysis (DFA)

	Coding used in DFA					
	Number of groups					
Bacterial taxon	17	22A	22B	25A	25B	35
Acinetobacter calcoaceticus va. lwoffii	10	13	14	17	17	24
Alcaligenes faecalis	11	14	15	18	18	25
Alcaligenes bronchisepticus	11	15	16	19	19	26
Flavobacterium meningosepticum	12	16	17	20	20	27
Enterobacter aerogenes	13	17	18	21	21	28
Enterobacter cloacae	13	18	19	22	22	29
Enterbacter agglomerans	13	17	18	21	21	30
Hafnia alvei	14	19	20	23	23	31
Salmonella	15	20	0	0	0	32
Edwardsiella tarda	16	21	21	24	24	33
Aeromonas hydrophila	17	22	22	25	25	34
Aeromonas formicans	17	22	22	25	25	35

Each grouping was subjected to DFA using all 26 tests (variables) and a number of restricted sets of variables, to determine the best mathematical treatment of the data.

The percentage agreements with previous identification obtained with the Training and Test Sets in the various combinations are shown in Table 7 when all 26 variables were included.

TABLE 7

Effect of grouping of taxa in the DFA on identification performance using all 26 variables

	Training set		Test set	
Number of groups	Number of strains	Agreement %	Number of strains	Agreement %
17	215	74.5	64	45.2
22A	215	78.9	64	45.9
22B	215	80.9	68	47.2
25A	221	81.4	52	50.0
25B	215	82.3	58	48.3
35	215	70.3	64	56.9

Highest percent of agreement was obtained in the training set with the 25B grouping and with the test set with 35 grouping. The effect on agreement percentage of exclusion of selected variables from the DFA is shown in Tables 8 and 9, using 22B and 25B groupings. "Variables" means the enzyme tests (a) to (z).

TABLE 8
Effect of excluding selected variables in th DFA on identification performance, using 22B grouping

Number of variables	Agreement %	Variables excluded, in addition t those excluded in the preceding line
26	80.9	—
25	81.4	PNAG
24	80.5	VP
23	75.8	INDOLE
22	76.3	PNAA
21	74.4	Glutamate decarboxylase
20	73.0	PNPA
15	67.4	Arg-NAP, leu-NAP, pro-NAP, beta-glucoronidase, DNAase
10	51.2	OH-pro-NAP, leu-4-m-NAP, pyr-NAP, alpha-glucosidase, beta-glucosidase

TABLE 9(a)
Effect of excluding selected variables in the DFA on identification performance, using 25B grouping

Number of strains	Bacterial genus	26	25	24	23	23
		Number of variables				
		% agreement				
23	*Escherichia*	95.6	91.3	91.3	91.3	91.3
24	*Klebsiella*	87.5	87.5	87.5	87.5	83.3
26	*Proteus*	69.2	65.4	65.4	65.4	65.4
17	*Pseudomonas*	94.1	94.1	94.1	94.1	94.1
19	*Staphylococcus*	84.2	84.2	78.9	78.9	78.9
7	*Streptococcus*	85.7	85.7	85.7	85.7	85.7
13	*Citrobacter*	84.6	84.6	84.6	84.6	84.6
17	*Providencia*	94.2	94.2	94.2	82.3	94.2
21	*Serratia*	71.4	71.4	71.4	66.6	61.9
10	*Acinetobacter*	70.0	80.0	70.0	70.0	60.0
2	*Alcaligenes*	100	100	100	100	100
22	*Flavobacterium*	100	100	100	100	100
22	*Enterobacter*	68.2	68.2	63.6	63.6	63.6
5	*Hafnia*	100	100	100	100	100
6	*Edwardsiella*	100	100	100	100	100
9	*Aeromonas*	77.8	77.8	77.8	77.8	77.8
	All genera	83.8	84.8	82.0	81.4	81.0
228	Tests excluded	NONE	PNAG	PNAG VP	PNAG VP PNPA	PNAG alpha-glucosidase beta-xylosidase

TABLE 9(b)
Effect of number of tests on % agreement with conventional identification

Percentage agreement	26	25	24	23	23
	Number of variables				
	Number of genera in each category				
90—100	7	7	7	6	7
80—89.9	4	5	3	4	3
70—79.9	3	2	4	3	2
60—69.9	2	2	2	3	4

In both cases exclusion of the PNAG test results in a slight increase in the total percentage agreement as well as in the number of genera showing 80% agreement. It is believed that this was due to experimental inaccuracies in the results arising from the PNAG test. Exclusion of further tests whether by computer selecti n or by inspection causes further decrease in the % of agreement which is unacceptable.

The taxa which proved difficult to separate are *Proteus rettgeri, Acinetobacter anitratus*, and the *Enterobacter/Serratia* species. This is probably a result of use of strains kept for a long time in culture.

Som of these strains when tested by both the conventional API 20 and R/B tub kits did not show 'good identification' and in a number of strains the results of one system did not agree with those of the second.

More strains, preferably freshly isolated ones, are needed f r solving the difficulties in these taxa.

The results obtained show, however, that the full procedure of the invention gives greater than 80% accuracy of identification over a very wide range of difficult bacteria. It is believed that this accuracy can be increased if enzyme profile information from a large and more representative group of previously identified bacteria is available for comparison.

Claims

1. A procedure for use in the identification of bacteria, in which bacteria are subjected to a combination of tests for the determination of bacterial enzymes, characterised in that tests are carried out for the quantitative determination of each of the following enzymes (a)—(z):—

(a) lipase
(b) alpha-glucosidase
(c) beta-glucosidase
(d) beta-xylosidase
(e) beta-glucuronidase
(f) beta-galactosidase
(g) whole cell acid phosphatase
(h) acid phosphatase in the presence of agents which disrupt the bacterial cell permeability barrier
(i) DL-alanyl-beta-naphthylamine specific peptidase
(j) L-arginyl-beta-naphthylamine specific peptidase
(k) N-gamma-L-glutamyl-beta-naphthylamine specific peptidase
(l) glycyl-beta-naphthylamine specific peptidase
(m) L-4-hydroxyprolyl-beta-naphthylamine specific peptidase
(n) L-leucyl-beta-naphthylamine specific peptidase
(o) L-leucyl-4-methoxy-beta-naphthylamine specific peptidase
(p) L-lysyl-beta-naphthylamine specific peptidase
(q) L-prolyl-beta-naphthylamine specific peptidase
(r) L-pyrrolidonyl-beta-naphthylamine specific peptidase
(s) alanyl-p-nitroaniline specific peptidase
(t) glutamyl-p-nitroaniline specific peptidase
(u) diacetyl/acetoin-producing enzymes.
(v) p-nitrophenylalanine ammonia-lyase
(w) tryptophanase
(x) deoxyribonuclease
(y) glutamate decarboxylate
(z) cytochrome oxidase,

in that the tests include incubation of the bacterial sample, with substrates for the enzymes, for a period of time which, except in the case of tryptophanase and deoxyribonuclease, is suitable for the determination of constitutive enzymes, and in that the tests, with the same exceptions, do not depend on growth of the bacteria during the test.

2. A procedure for use in the rapid differentiation of bacteria of the groups *Escherichia, Klebsiella spp, Proteus spp* and *Pseudomonas spp*, in which a sample comprising bacteria of one of these groups is subjected to a combination of tests for determination of bacterial enzymes, characterised in that tests are carried out for the quantitative determination of each of the following enzymes: acid phosphatase in the presence of an agent which disrupts the bacterial cell permeability barrier, beta-galactosidase, glutamate decarboxylase, phenylalanine deaminase, cytochrome oxidase, diacetyl/acetoin-producing enzymes and urease, in that the tests include incubation of the bacterial sample, with substrates for the enzymes, for a period of time suitable for the determination of constitutive enzymes, and in that the tests do not depend on growth of the bacteria during the test.

3. A procedure according to Claim 1, characterised in that the period of incubation is about 2 hours except for the tryptophanase and deoxyribonuclease tests (w) and (x).

4. A procedure according to Claim 2, characterised in that the period of incubation is about 2 hours.

5. A procedure according to Claim 1 or 3 characterised in that the tryptophanase and deoxyribonuclease tests (w) and (x) are carried out with a period of incubation of 2 to $2\frac{1}{2}$ hours.

6. A procedure according to any preceding claim characterised in that bacterial cultures are prepared from clinical specimens and colonies of bacteria to be identified are harvested from the

cultures after a sufficient period of growth and made up into a suitable form for determination by the procedure of said preceding claim.

7. A procedure according to any one of Claims 1 to 5, characterised in that it is carried ut on a sample derived from a single colony of bacteria produced by pre-cultur of a clinical specimen.

8. A procedure according to any of the preceding claims, characterised in that incubation during the tests is carried out at a temperature of about 40°C.

9. A procedure according to any of the preceding claims, characterised in that a single procedure, using only the defined combination of tests, is used for identification of the bacterial sample.

10. A procedure according to any of the preceding claims characterised in that continuous flow analysis techniques are used.

11. A procedure according to any of Claims 1 to 9 characterised in that it is carried out using apparatus comprising a plurality of compartments which separately contain specific enzyme substrates and in that sample is added to each compartment and the enzymes are quantitatively determined by the measurement of products in the compartments, using other reagents as required.

12. A kit comprising reagents for use in a procedure according to Claim 1 or 2 characterised by comprising separate specific substrates for each of the enzymes defined in Claim 1 or 2.

13. A kit according to Claim 12, characterised in that the enzyme substrates give rise to products which may be measured by fluorimetry or colorimetry.

14. A kit according to Claim 13 characterised in that it includes colour-developing or fluorescence-developing reagent(s).

15. A kit according to Claim 12, 13 or 14, characterised by comprising suitable buffers and other reagents.

16. A kit according to any of Claims 12 to 15, characterised by comprising suitable apparatus comprising a plurality of wells or compartments which separately contain specific enzyme substrates for each of the enzyme tests of the procedure and which is adapted to automated spectrometric scanning techniques.

Patentansprüche

1. Verfahren zur Anwendung bei der Identifizierung von Bakterien wobei Bakterien einer Kombination von Tests zur Bestimmung von bakteriellen Enzymen unterworfen werden, dadurch gekennzeichnet, daß die Tests für die Bestimmung jedes der folgenden Enzyme (a) bis (z) durchgeführt werden:

(a) Lipase
(b) α-Glucosidase
(c) β-Glucosidase
(d) β-Xylosidase
(e) β-Glucuronidase
(f) β-Galactosidase
(g) saure Phosphatase der Gesemtzelle
(h) saure Phosphatase in Gegenwart von Mitteln, welche die Permeabilitätsbarriere der Bakterienzelle zerstören
(i) D L-Alanyl-β-naphthylamin spezifische Peptidase
(j) L-Arginyl-β-naphthylamin spezifische Peptidase
(k) N-γ-L-Glutamyl-β-naphthylamin spezifische Peptidase
(l) Glycyl-β-naphthylamin spezifische Peptidase
(m) L-4-Hydroxyprolyl-β-naphthylamin spezifische Peptidase
(n) L-Leucyl-β-naphthylamin spezifische Peptidase
(o) L-Leucyl-4-methoxy-β-naphthylamin spezifische Peptidase
(p) L-Lysyl-β-naphthylamin spezifische Peptidase
(q) L-Prolyl-β-naphthylamin spezifische Peptidase
(r) L-Pyrrolidonyl-β-naphthylamin spezifische Peptidase
(s) Alanyl-p-nitroanilin spezifische Peptidase
(t) Glutamyl-p-nitroanilin spezifische Peptidase
(u) Diacetyl/Acetoin produzierende Enzyme
(v) p-Nitrophenylalanin Ammoniak-Lyase
(w) Tryptophanase
(x) Desoxyribonuclease
(y) Glutamatdecarboxylase
(z) Cytochromoxidase

wobei die Tests die Inkubation der Bakterienprobe mit Substraten für die Enzyme für eine Zeitspanne umfassen, die sich mit Ausnahme von Tryptophanase und Desoxyribonuclease für die Bestimmung der

jeweiligen Enzyme eign t und daß di Tests, mit den gleichen Ausnahmen, nicht vom Wachstum der Bakterien während des Tests abhängen.

2. Verfahren zur Anwendung bei der raschen Differenzi rung von Bakterien der Gruppen *Escherichia, Klebsiella spp., Proteus* spp. und *Pseudomonas spp.*, wobei eine Bakterien einer dieser Gruppen enthaltende Prob einer Kombination von Tests zur Bestimmung von Bakterienenzymen unterworfen wird, dadurch gekennzeichnet, daß die Tests zur Bestimmung von jedem der folgenden Enzyme durchgeführt werden: sauere Phosphatase in Gegenwart eines Mittels, welches die Permeabilitätsbarriere der Bakterienzelle zerstört, beta-Galactosidase, Glutamatdecarboxylase, Phenylalanindeaminase, Cytochromoxidase, Diacetyl/Acetoin-erzeugende Enzyme und Urease, und daß die Tests die Inkubation der Bakterienprobe mit Substraten für die Enzyme für eine Zeitspanne umfassen, die sich zur Bestimmung der jeweiligen Enzyme eignet und daß die Tests nicht vom Wachstum der Bakterien während des Tests abhängen.

3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Inkubationsdauer etwa 2 Stunden beträgt, ausgenommen für die Tryptophenase- und Desoxyribonuclease-Tests (w) und (x).

4. Verfahren nach Anspruch 2, dadurch gekennzeichnet, daß die Inkubationsdauer etwa 2 Stunden beträgt.

5. Verfahren nach Anspruch 1 oder 3, dadurch gekennzeichnet, daß die Tryptophenase- und Desoxyribonuclease-Tests (w) und (x) mit einer Inkubationsdauer von 2 bis 2 1/2 Stunden durchgeführt werden.

6. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß Bakterienkulturen aus klinischen Proben hergestellt und Kolonien der zu identifizierenden Bakterien von den Kulturen nach ausreichender Wachstumszeit geerntet und in eine geeignete Form für die Bestimmung nach dem Verfahren eines der vorhergehenden Ansprüche überführt werden.

7. Verfahren nach einem der vorhergehenden Ansprüche 1 bis 5, dadurch gekennzeichnet, daß es an einer Probe durchgeführt wird, die aus einer Einzelkolonie von Bakterien stammt, die durch Vorkultur einer klinischen Probe erzeugt wurde.

8. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die Inkubation während der Tests bei einer Temperatur von etwa 40°C durchgeführt wird.

9. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß eine einzige Arbeitsweise unter Verwendung nur der definierten Kombination von Tests zur Identifizierung der Bakterienprobe angewandt wird.

10. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß kontinuierliche Fließanalysen-Arbeitsweisen angewandt werden.

11. Verfahren nach einem der Ansprüche 1 bis 9, dadurch gekennzeichnet, daß es durchgeführt wird unter Verwendung einer Apparatur, die eine Mehrzahl von Abteilungen umfaßt, die getrennt spezifische Enzymsubstrate enthalten und daß die Probe in jedes Abteil eingebracht wird und die Enzyme quantitativ durch die Messung von Produkten in den Abteilungen bestimmt werden, wobei andere Reagenzien nach Erforderniss verwendet werden.

12. Kit, enthaltend Reagenzien zur Verwendung in einem Verfahren gemäß Anspruch 1 oder 2, dadurch gekennzeichnet, daß es getrennte spezifische Substrate für jedes der in Anspruch 1 oder 2 definierten Enzyme aufweist.

13. Kit nach Anspruch 12, dadurch gekennzeichnet, daß das Enzymsubstrat Anlaß zu Produkten gibt, die durch Fluorimetrie oder Colorimetrie gemessen werden können.

14. Kit nach Anspruch 13, dadurch gekennzeichnet, daß es eines oder mehrere farbentwickelnde oder Fluoreszenz entwickelnde Reagenzien umfaßt.

15. Kit nach Anspruch 12, 13 oder 14, dadurch gekennzeichnet, das es geeignete Puffer und andere Reagenzien umfaßt.

16. Kit nach einem der Ansprüche 12 bis 15, dadurch gekennzeichnet, daß es eine geeignete Apparatur enthält, die eine Mehrzahl von Mulden oder Abteilungen aufweist, die getrennt spezifische Enzymsubstrate für jeden der Enzymtests des Verfahrens aufweisen und diese der automatisierten spektrometrischen Abtasttechnik angepaßt ist.

Revendications

1. Procédé à utiliser pour l'identification de bactéries, suivant lequel les bactéries sont soumises à une combinaison de tests pour la détermination des enzymes bactériennes, caractérisée en ce que des tests sont exécutés pour la détermination de chacune des 26 enzymes (a)—(z) suivantes:

(a) lipase
(b) α-glucosidase
(c) β-glucosidase
(d) β-xylosidase
(e) β-glucuronidase
(f) β-galactosidase
(g) phosphatase acide de cellule entière

(h) phosphatase acide en présence d'agents qui rompent la barrière de perméabilité cellulaire bactérienne
(i) peptidase spécifique de la DL-analyl-β-naphtylamine
(j) peptidase spécifique d la L-arginyl-β-naphtylamine
(k) peptidase spécifique de la N-γ-L-glutamyl-β-naphtylamine
(l) peptidase spécifique de la glycyl-β-naphtylamine
(m) peptidase spécifique de la L-4-hydroxyprolyl-β-naphtylamine
(n) peptidase spécifique de la L-leucyl-β-naphtylamine
(o) peptidase spécifique de la L-leucyl-4-méthoxy-β-naphtylamine
(p) peptidase spécifique de la L-lysyl-β-naphtylamine
(q) peptidase spécifique de la L-prolyl-β-naphtylamine
(r) peptidase spécifique de la L-pyrrolidonyl-β-naphtylamine
(s) peptidase spécifique de alanyl-p-nitroaniline
(t) peptidase spécifique de la glutamyl-p-nitroaniline
(u) enzymes produisant la diacétyle/acétoïne
(v) p-nitrophénylalanine ammonia-lyase
(w) tryptophanase
(x) désoxyribonucléase
(y) glutamate décarboxylase
(z) cytochrome oxydase,

en ce que les tests comprennent l'incubation de l'échantillon bactérien, avec des substrats pour les enzymes, pendant une durée qui, sauf dans le cas de la tryptophanase et de la désoxyribonucléase, est appropriée pour la détermination des enzymes constitutives, et en ce que les tests, avec les mêmes exceptions, ne dépendent pas du développement des bactéries pendant le test.

2. Procédé à utiliser pour la différenciation rapide de bactéries des groupes *Escherichia, Klebsiella spp., Proteus spp* et *Pseudomonas spp.*, suivant lequel un échantillon comprenant des bactéries de l'un de ces groupes est soumis à une combinaison de tests pour la détermination des enzymes bactériennes, caractérisé en ce que des tests sont exécutés pour la détermination quantitative de chacune des enzymes suivantes: phosphatase acide en présence d'un agent qui rompt la barrière de perméabilité cellulaire bactérienne, β-galactosidase, glutamate, décarboxylase, phénylalanine désaminase, cytochrome oxydase, enzymes produisant de la diacétyle/acétoïne et uréase, en ce que les tests comprennent l'incubation de l'échantillon bactérien, avec des substrats pour les enzymes, pendant une durée appropriée pour la détermination des enzymes constitutives, et en ce que les tests ne dépendent pas du développement des bactéries pendant le test.

3. Procédé suivant la revendication 1, caractérisé en ce que la durée d'incubation est d'environ 2 heures sauf pour les tests (w) et (x) de la tryptophanase et de la désoxyribonucléase.

4. Procédé suivant la revendication 2, caractérisé en ce que la durée d'incubation est d'environ 2 heures.

5. Procédé suivant la revendication 1 ou 3, caractérisé en ce que les tests (w) et (x) de la tryptophanase et de la désoxyribonucléase sont exécutés avec une durée d'incubation de 2 à $2\frac{1}{2}$ heures.

6. Procédé suivant l'une quelconque des revendications précédentes, caractérisé en ce que des cultures bactériennes sont préparées à partir de prélèvements cliniques et des colonies de bactéries à identifier sont collectées à partir des cultures après une durée de développement suffisante et sont amenées sous une forme propre à la détermination par le procédé de la dite revendication précédente.

7. Procédé suivant l'une quelconque des revendications 1 à 5, caractérisé en ce qu'il est exécuté sur un échantillon issu d'une seule colonie de bactéries produite par préculture d'un prélèvement clinique.

8. Procédé suivant l'une quelconque des revendications précédentes, caractérisé en ce que l'incubation pendant les tests est exécutée à une température d'environ 40°C.

9. Procédé suivant l'une quelconque des revendications précédentes, caractérisé en ce qu'on applique un procédé unique, mettant en jeu uniquement la combinaison de tests définie, pour l'identification de l'échantillon bactérien.

10. Procédé suivant l'une quelconque des revendications précédentes, caractérisé en ce qu'on utilise des techniques d'analyse en écoulement continu.

11. Procédé suivant l'une quelconque des revendications 1 à 9, caractérisé en ce qu'il est exécuté au moyen d'un appareil comprenant plusieurs compartiments qui contiennent séparément des substrats spécifiques des enzymes et en ce que de l'échantillon est introduit dans chaque compartiment et les enzymes sont déterminées quantitativement par la mesure de produits dans les compartiments, au moyen d'autres réactifs suivant les besoins.

12. Trousse comprenant des réactifs à utiliser dans un procédé suivant la revendication 1 ou 2, caractérisé en ce qu'elle comprend des substrats spécifiques séparés pour chacune des enzymes définies à la revendication 1 ou 2.

13. Trousse suivant la revendication 12, caractérisée en c que les substrats pour les enzymes donnent des produits qui peuvent ˆtre mesur´s par fluorimétrie ou colorimétrie.

14. Trousse suivant la revendication 13, caractérisé en ce qu'elle comprend un ou des réactifs de dévloppement chromogènes ou d développem nt fluorogènes.

15. Trousse suivant la revendication 12, 13 ou 14, caractérisé en ce qu'elle comprend les tampons et autres réactifs appropriés.

16. Trousse suivant l'une quelconque des revendications 12 à 15, caractérisée en ce qu'elle comprend un appareil approprié qui comprend plusieurs cuvettes ou compartiments qui contiennent séparément des substrats spécifiques des enzymes pour chacun des tests enzymatiques du procédé et qui convient pour les techniques d'exploration spectrométrique automatisées.

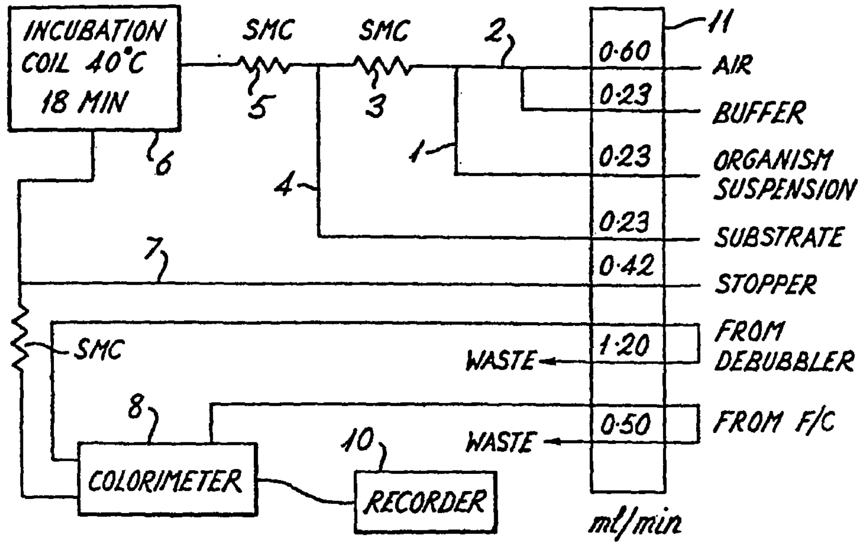
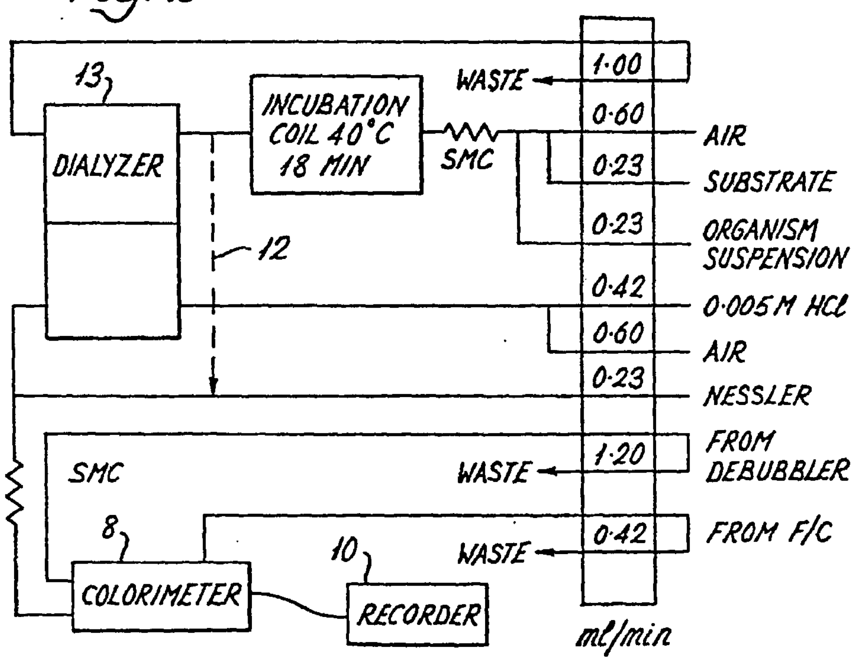

INCUBATION COIL 40°C 18 MIN
SMC
SMC
2
11
5
3
6
1
4
7
SMC
8
10
0·60
0·23
0·23
0·23
0·42
1·20
0·50
AIR
BUFFER
ORGANISM SUSPENSION
SUBSTRATE
STOPPER
FROM DEBUBBLER
FROM F/C
WASTE
WASTE
COLORIMETER
RECORDER
ml/min

Fig. 1

Fig. 2


13
DIALYZER
INCUBATION COIL 40°C 18 MIN
SMC
WASTE
12
SMC
8
10
1·00
0·60
0·23
0·23
0·42
0·60
0·23
1·20
0·42
AIR
SUBSTRATE
ORGANISM SUSPENSION
0·005M HCl
AIR
NESSLER
FROM DEBUBBLER
FROM F/C
WASTE
WASTE
COLORIMETER
RECORDER
ml/min

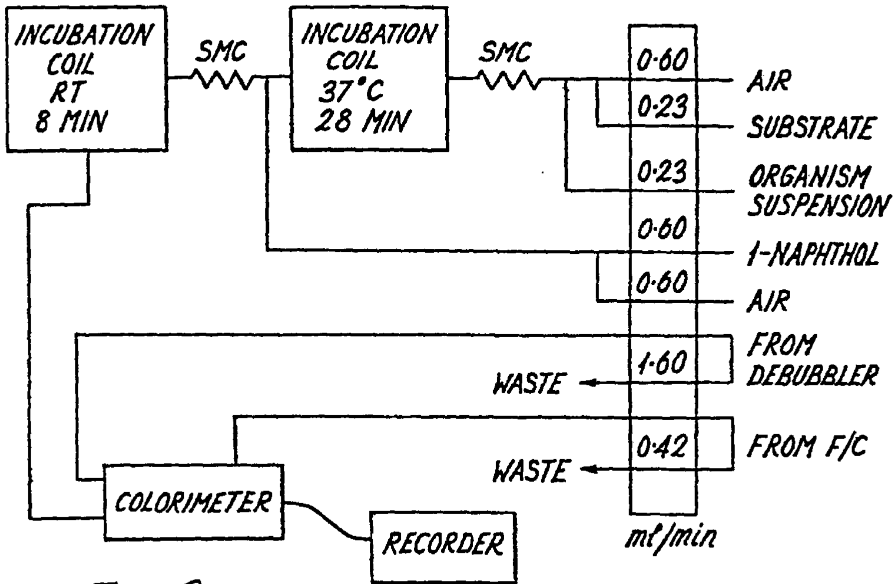

INCUBATION COIL RT 8 MIN
SMC
INCUBATION COIL 37°C 28 MIN
SMC
0·60
0·23
0·23
0·60
0·60
1·60
0·42
AIR
SUBSTRATE
ORGANISM SUSPENSION
1-NAPHTHOL
AIR
FROM DEBUBBLER
FROM F/C
WASTE
WASTE
COLORIMETER
RECORDER
ml/min

Fig. 3

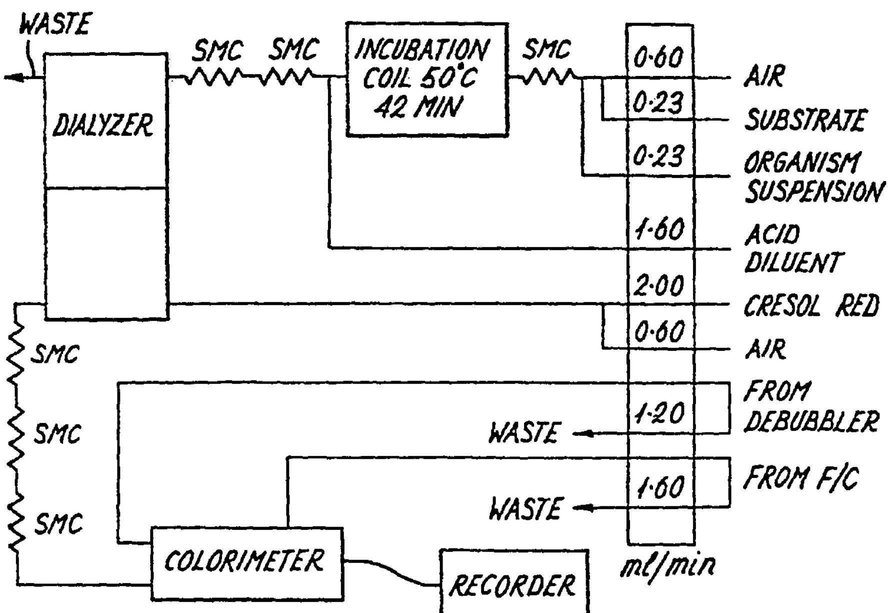

WASTE
DIALYZER
SMC
SMC
INCUBATION COIL 50°C 42 MIN
SMC
0·60
0·23
0·23
1·60
2·00
0·60
1·20
1·60
AIR
SUBSTRATE
ORGANISM SUSPENSION
ACID DILUENT
CRESOL RED
AIR
FROM DEBUBBLER
FROM F/C
WASTE
WASTE
SMC
SMC
SMC
COLORIMETER
RECORDER
ml/min

Fig. 4

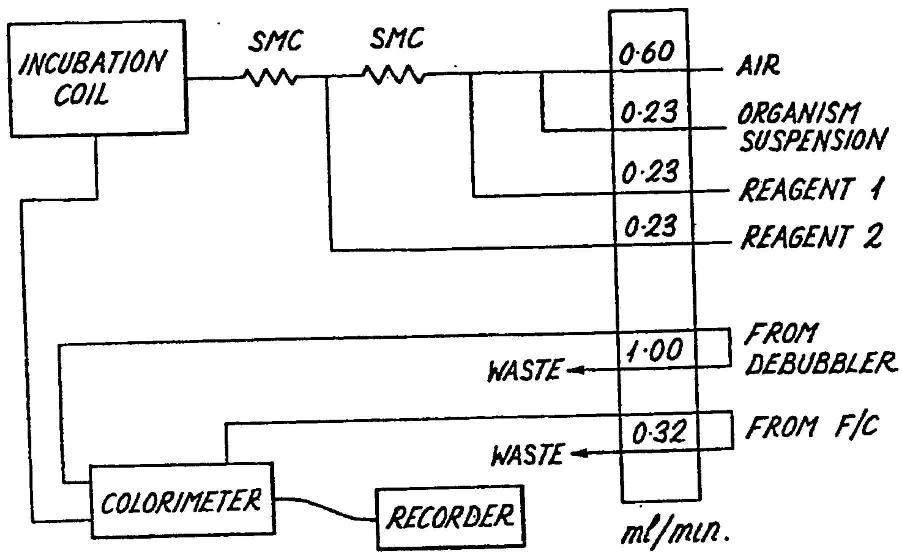
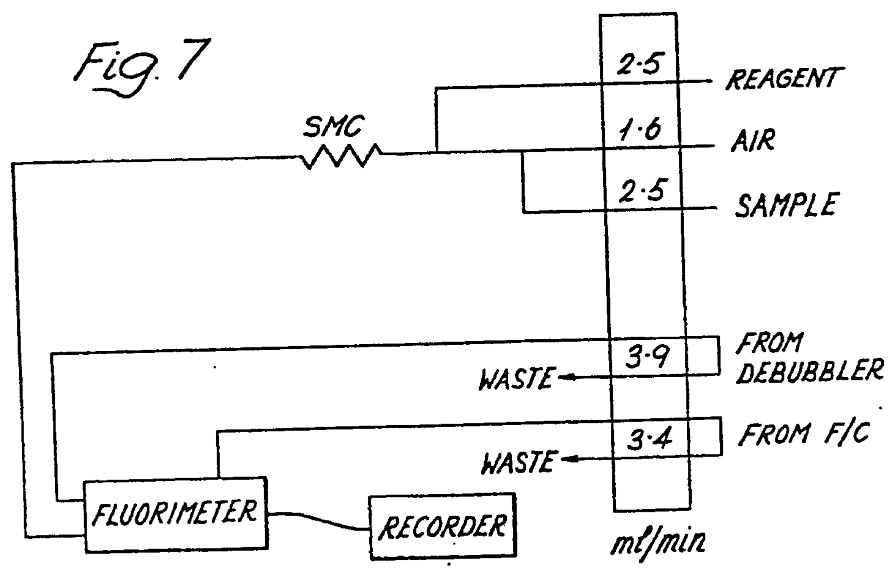

INCUBATION COIL
SMC
SMC
0·60
AIR
0·23
ORGANISM SUSPENSION
0·23
REAGENT 1
0·23
REAGENT 2
1·00
FROM DEBUBBLER
WASTE
0·32
FROM F/C
WASTE
COLORIMETER
RECORDER
ml/min.

Fig. 5

Fig. 7


SMC
2·5
REAGENT
1·6
AIR
2·5
SAMPLE
3·9
FROM DEBUBBLER
WASTE
3·4
FROM F/C
WASTE
FLUORIMETER
RECORDER
ml/min

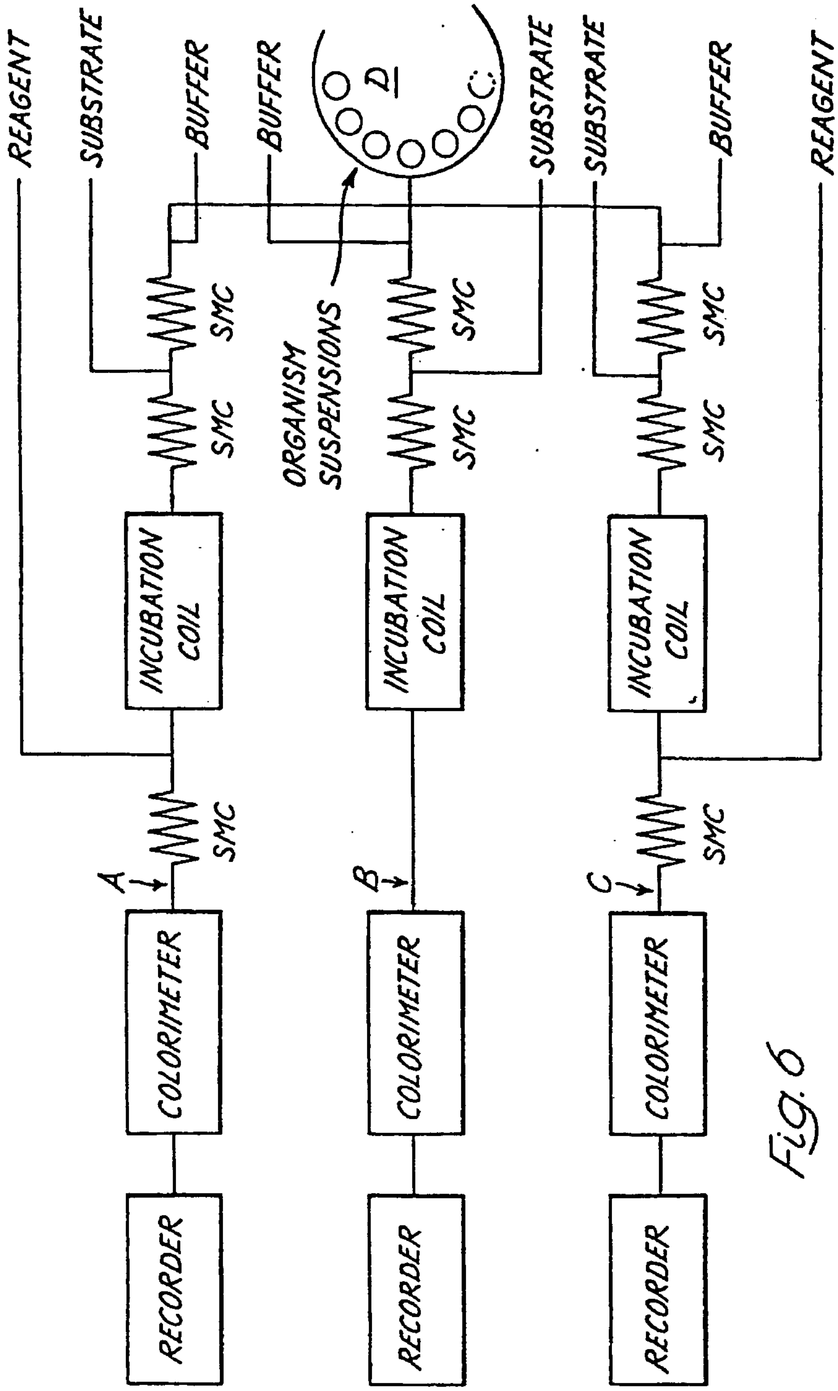

REAGENT
SUBSTRATE
BUFFER
BUFFER
D
SUBSTRATE
SUBSTRATE
BUFFER
REAGENT
SMC
SMC
SMC
SMC
SMC
SMC
ORGANISM SUSPENSIONS
INCUBATION COIL
INCUBATION COIL
INCUBATION COIL
SMC
SMC
A
B
C
COLORIMETER
COLORIMETER
COLORIMETER
RECORDER
RECORDER
RECORDER

Fig. 6